

## ISOLATION OF CYSTEINE AND METHIONINE AUXOTROPHIC MUTANTS IN *SINORHIZOBIUM MELILOTI*

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### ABSTRACT

Random Tn5 mutagenesis produced 21 cysteine/methionine and 13 methionine auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd201. Linkage of Tn5 to auxotroph indicated that each mutant had a single Tn5 insertion. All auxotroph showed spontaneous reversion to prototrophy and they resembled the parental strain in production of cell surface molecule and utilization of sugars and dicarboxylic acid.

### INTRODUCTION

Nitrogen fixing bacteria like *Klebsiella pneumoniae* and *Azotobacter* are able to reduce atmospheric nitrogen into ammonia as free living organisms. Other organisms fix nitrogen only in symbiotic relationship with an eukaryotic host plant, like rhizobia in legume symbiosis (1). The detail knowledge of the gene involved in symbiosis will be of great help in obtaining symbiotic combinations having enhanced ability of nitrogen fixation.

Rhizobial cell surface molecules which play an important role in infection of the legume host plant, is composed of exopolysaccharides (EPS), lipopolysaccharides (LPS),  $\beta$ -glucans and capsular polysaccharides (KPS). Normal expression of the genes for cell surface molecules is required for rhizobium-legume symbiosis (2,3,4,5,6).

Sulfur-containing amino acids auxotrophs of rhizobia have been isolated by several workers (7,8,9). Methionine and cysteine/methionine mutants of *S. meliloti* strain 2011 were found to induce effective nodules on alfalfa plant (10). Cysteine-requiring mutants of *S. meliloti* strain L5-30 showed loss of effectiveness (11). Kerppola and Kahn (12) observed that the methionine mutants of *S. meliloti* strain 104A14 formed ineffective nodule on alfalfa. *Rhizobium etli* *metZ* mutant was unable to produce lipochito-oligosaccharides or induce nodules on root of *Phaseolus vulgaris* (13).

### MATERIAL AND METHODS

**Strains and plasmids:** The bacterial strains and plasmid used in this study are listed in table 1.

**Media and supplements:** Complete medium trypton yeast extract (TY) and rhizobial minimal medium (RMM) have been described earlier (14). Stock solutions were added to the autoclaved media to make final concentrations of 50, 30 and 10 mg/ml from amino acids, nitrogen bases and vitamins, respectively.

Antibiotics used were purchased from HiMedia Laboratories. Streptomycin sulfate and kanamycin acid sulfate stock solutions were prepared in sterile distilled water. The final concentration of antibiotics used in different media were as follows: streptomycin sulfate (100  $\mu$ g/ml; kanamycin acid sulfate (100  $\mu$ g/ml for *E. coli* and

400 µg/ml for *S. meliloti*), Chloramphenicol (50 µg/ml), rifampicin (40 µg/ml) and tetracycline hydrochloride (15 µg/ml).

**Transposon Tn5 mutagenesis:** Bacterial conjugations were done according to Kondorosi *et al* (15). Random mutagenesis of *S. meliloti* strain Rmd 201 was carried out using Tn5 delivery suicide plasmid pGS9 (16). Transconjugants obtained were screened for auxotrophs by streaking them on RMM and TY+Km<sup>400</sup>+Sm<sup>100</sup> agar medium. The auxotrophy of each strain was determined on the modified Holliday pools (17).

**Linkage of Tn5 insertion to auxotrophy:** Transposon Tn5 encoded kanamycin resistance marker from each auxotroph was mobilized to *S. meliloti* ZB557 strain with the help of genome mobilizing plasmid pJB3J1.

**Reversion analysis of auxotrophs:** Spontaneous reversion frequency for the auxotroph was calculated on the basis of the number of prototrophic revertants (number of colonies on RMM) and the total number of cell (number of colonies on TY medium), after streaking the same volume on each medium.

**Test for production of cell surface molecules:** The test for production of lipopolysaccharides, the test for production of succinylated exopolysaccharides, the test for production of cellulose fibrils, the test for production of β-(1-3)-glucans and the test for production of β-(1-2)-glucans were done as describe earlier (18).

## RESULTS AND DISCUSSION

**Isolation of auxotrophs:** Three thousand transposon Tn5 derivatives (Km<sup>r</sup>) of *S. meliloti* strain Rmd201 were obtained and out of these 12 did not grow on *Rhizobium* minimal medium (RMM). Three sulfur amino acid auxotrophs isolated during this study and two such auxotrophs generated previously by other workers were used for further studies. The auxotrophic mutants with their requirements were listed in table 2. Isolation of auxotrophs which grow on cysteine or methionine has also been reported by several workers (7,13). Methionine auxotroph cannot grow on RMM supplemented with cysteine. It seems that *S. meliloti* cannot convert cysteine to methionine.

**Linkage of Tn5 insertion to auxotrophy:** All kanamycin resistant transconjugants showed the respective donors auxotrophic marker, hence, there was 100% cotransfer of Tn5 and the auxotrophic marker. This indicate that the Tn5 insertion is responsible for auxotrophy in each strain.

**Reversion analysis:** All auxotrophic mutants showed spontaneous reversion to prototrophy with a percentage range between  $4.5 \times 10^{-8}$  to  $4.5 \times 10^{-6}$  (table 3). The revertants did not grow on TY medium containing kanamycin. This indicates the excision of transposon Tn5 from the genome.

### **Production of cell surface molecules:**

The results of production of cell surface molecule are listed in table 4. All auxotrophic mutants, like the parental strain Rmd201, produced lipopolysaccharides (LPS), succinylated exopolysaccharides (EPSI), cellulose fibrils, β-(1-2)-glucan and β-(1-3)-glucan. It seems that the genes involved in cysteine and methionine synthesis is not responsible for cell surface molecule production.

### **Utilization of sugars and dicarboxylic acids:**

No change in the growth behavior of any auxotroph was detected when glucose in RMM was replaced by any of the other sugars (arabinose, maltose, sorbitol, manitol, or sucrose) or dicarboxylic acids (malic acid, aspartic acid or sodium succinate) as carbon source. The presence of functional C<sub>4</sub>-dicarboxylic acid transport system of *R. leguminosarum* was found to be essential for nitrogen fixation (19).

**Table 1:** Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	source/reference
<b><i>Sinorhizobium meliloti</i></b>		
AK631	Nod+Fix+, compact colony variant of wild type Rm41	Adam Kondorosi
Rmd201	spontaneous Sm <sup>r</sup> derivatives of AK631	Khanuja &kumar (20)
PP631	AK631(pJB3J1)	Peter Putnoky
ZB557	Rm41 <i>Phe15 leu4 rfl sm1</i>	Peter Putnoky
BA4, BA7	Rmd201 <i>cysI/cysJ::Tn5</i>	This study
BA8	Rmd201 <i>metA/metZ::Tn5</i>	This study
VK29	Rmd201 <i>metE::Tn5</i>	Vineetha KE & Prasad CK
VK39	Rmd201 <i>metF::Tn5</i>	Vineetha KE & Prasad CK
<b><i>Escherichia coli</i></b>		
WA803 (pGS9)	Met <sup>-</sup> Thi <sup>-</sup> Cmr Kmr	Selvaraj & Iyer (16)
<b>Plasmids</b>		
PGS9	IncN rep15A Cmr Kmr	Selvaraj & Iyer (16)
PJB3J1	Kms derivative of pR68.45, capable of mobilizing genomic segments of its host, Ter Cbr Nal <sup>r</sup>	Brewin <i>et al.</i> (21)

**Table 2.** Auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd21 and their nutritional requirements.

Strain	Nutritional requirement
BA4	Cysteine or methionine
BA7	Cysteine or methionine
BA8	Methionine
VK29	Methionine
VK39	Methionine

**Table 3.** Reversion frequencies of auxotrophic mutants of *Sinorhizobium meliloti* Rmd201.

<i>S. meliloti</i> strain	Reversion percentage
BA4	$4.5 \times 10^{-8}$
BA7	$3.7 \times 10^{-8}$
BA8	$3.2 \times 10^{-8}$
VK29	$3.7 \times 10^{-8}$
VK39	$4.5 \times 10^{-6}$

**Table 4.** Production of cell surface molecules by *Sinorhizobium meliloti* parental strain Rmd201 and its auxotrophic mutants.

Strain	LPS	EPSI	Cellulose Fibrils	$\beta$ -1-2-gluan	$\beta$ -1-3-glucon
Rmd201	+	+	+	+	+
BA4	+	+	+	+	+
BA7	+	+	+	+	+
BA8	+	+	+	+	+
VK29	+	+	+	+	+
VK39	+	+	+	+	+

عزل طافرات العوز الغذائي الاميني السستين والمثيونين في بكتريا السينورايذوبيوم ميليلوتي

ياسل عبد الزهرة عباس

كلية الطب البيطري ، جامعة البصرة ، البصرة

#### الخلاصة

باستخدام الترانسفوسون Tn5 تم الحصول على ٢١ طافر للحامض الاميني سبستين او مثيونين و ١٣ طافر للحامض الاميني مثيونين في بكتريا سينورايذوبيوم ميليلوتي السلالة Rmd201 . لقد اكد ارتباط العوز الغذائي للطافر مع Tn5 ادخال Tn5 واحد الى DNA البكتريا . بينت الدراسة ان كل الطافرات كان لها قابلية الارتداد الى الوضع الطبيعي وكانت الطفرات متشابهة للسلالة الابوية في انتاج بعض المواد السطح خلوية وكذلك في استغلال بعض السكريات والاحماض ثنائية الكربوكسيل .

#### REFERENCES

- (1) Van Kammen , A. 1995. The molecular development of nitrogen fixing root nodules. In: Nitrogen Fixation: Fundamental and Application. I.A. Tikhonovich *et al.* (eds.).
- (2) Carlson, R.W., Bhat, U.R. and Reuhs, B. 1992. *Rhizobium* lipopolysaccharides: Their structures and evidence for their importance in the nitrogen-fixing symbiotic infection of their host legumes. In: Plant biotechnology and development, P.M. Gresshoff (ed.), p:33-44. CRC Press, Boca Raton, Fla.
- (3) Jelpi, L., Dylan, T., Ditta, G.S., Helinski, D.R. and Stanfield, S.W. 1990. The *ndvB* locus of *Rhizobium meliloti* encodes a 319-kDa protein involved in the production of  $\beta$ -(1-2) glucan. J. Biol. Chem. 265:2843-2851.
- (4) Kereszt, A., Kiss, E., Reuhs, B.L., Carlson, R.W., Kondorosi, A. and Putnoky, P. 1998. Novel *rkp* gene clusters of *Sinorhizobium meliloti* involved in capsular

- polysaccharide production and invasion of the symbiotic nodule: The *rkpK* gene encodes a UDP-glucose dehydrogenase. J. Bacteriol. 180: 5426-5431.
- (5) Rolf, B.G., Carlson, R.W., Ridge, R.W., Dazzo, F.B., Mateos, P.F. and Pankhurst, C.E. 1996. Defective infection and nodulation of clovers by exopolysaccharides mutants of *Rhizobium leguminosarum* bv. *Trifolii*. Aust. J. plant Physiol. 23:285-303.
- (6) Skorupska, A., Bailek, U., Urbanik-Sypniewska, T. and Lammeren, A. 1995. Two Types of nodules induced on *Trifolium pratense* by mutants of *Rhizobium leguminosarum* bv. *Trifolii* deficient in exopolysaccharides production. J. Plant Physiol. 147:93-100.
- (7) Mead, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E. and Ausubel, F.M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by Tn5 mutagenesis. J. Bacteriol. 149: 114-122.
- (8) Pain, A.N. 1979. Symbiotic properties of antibiotic-resistant and auxotrophic mutants of *Rhizobium leguminosarum*. J. Appl. Bacteriol. 47:53-64.
- (9) Singh, A., Ram, J., Sikka, V.K. and Kumar S. 1984. Derivation of marked strains in *Rhizobium leguminosarum* Rld1 by nitrosoguanidine and transposon mutagenesis. Ind. J. Exp. Biol. 22:239-247.
- (10) Scherrer, A. and Denarie, J. 1971. Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti* and of their prototrophic revertants. Plant Soil (special vol.):39-45.
- (11) Malek, W. and Kowalski, M. 1977. Structure of nodules induced by auxotrophic and ineffective mutants of *Rhizobium meliloti* strain L5-30 requiring cysteine, arginine+uracil and histidine. Acta Microbiol. Pol. 32:19-24.
- (12) Kerppola, T.K. and Kahn, M.L. 1988. Symbiotic phenotypes of auxotrophic mutants of *Rhizobium meliloti* 104A14. J. Gen. Microbiol. 134:913-919.
- (13) Tate, R., Riccio, A., Caputa, E., Iaccarino, M. and Pateriarca, E.J. 1999. The *Rhizobium etli* *metZ* gene is essential for methionine biosynthesis and nodulation of *Phaseolus vulgaris*. Mol. Plant-Microbe Interact. 12:24-34.
- (14) Kim, C.-H., Kuykendall, L.D., Shah, K.S. and Keister, D.L. 1988. Induction of symbiotically defective auxotrophic mutants of *Rhizobium fredii* HH303 by transposon mutagenesis. Appl. Environ. Microbiol. 54:423-427.
- (15) Kondorosi, A., Kiss, G.B., Forrai, T. Vincze, E. and Banfalvi, Z. 1977. Circular linkage map of *Rhizobium meliloti* chromosome. Nature 268:525-527.
- (16) Selvaraj, G. and Iyer, V.N. 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. 156:1292-1300.
- (17) Holliday, R. 1956. A new method for identification of biochemical mutants of microorganisms. Nature 178:987.
- (18) Ali, I.A. 1999. Genetic and biochemical studies on stress tolerance in *Rhizobium*. Ph. D. thesis, IIT Roorkee, India.
- (19) Finan, T.M., Wood, J.M. and Jordan, D.C. 1983. Symbiotic properties of C<sub>4</sub>-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. J. Bacteriol. 154:1403-1413.
- (20) Khanuja, S.P.S. and Kumar, S. 1988. Isolation of phages for *Rhizobium meliloti* AK631. Indian J. Exp. Biol. 26:665-667.
- (21) Brewin, N.J., Beringer, J.E. and Johnston, A.W.B. 1980. Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120:413-420.